Abstract

In human brain, monoamine oxidase plays a major role in regulation of monoamine neurotransmitter level by the oxidative deamination. However, its occurrence in the outer membrane of mitochondria suggests that monoamine oxidase may be involved in the decision of cell death or survival of neurons, since the mitochondria are now considered to mediate death signal in neurodegenerative disorders, such as Parkinson's disease. Recently we examined the role of type A monoamine oxidase in apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol. The (*R*)enantiomers of salsolinol [1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] derivatives were found to inhibit type A monoamine oxidase in competition to the substrates. In addition, the selective neurotoxicity of the (*R*)enantiomer of *N*-methylated salsolinol to dopamine neurons was confirmed by preparation of an animal model of Parkinson's disease in rats. *N*-Methyl(*R*)salsolinol enantio-selectively binds to the outer membrane of mitochondria, induces mitochondrial permeability transition in isolated mitochondria, and causes apoptosis in cultured dopaminergic neurons. These results suggest the occurrence of the target protein with stereo-structure similar to the binding site of type A monoamine oxidase. On the other hand, among inhibitors of type B monoamine oxidase some propargylamine derivatives protect neuronal cells from apoptosis by stabilizing mitochondrial membrane potential and inhibiting the activation of cell death signal transduction. The anti-apoptotic function of the propargylamines depends on their stereo-chemical structure; the (*R*)-enantiomers protect neurons more markedly than the (*S*)-enantiomers, suggesting the presence of the distinct binding site in mitochondria. These results strongly suggest that monoamine oxidase or structurally related proteins may play a major role in the regulation of cell death and survival in neurodegenerative disorders.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder and its major pathological feature is the cell death of dopamine neurons in the substantia nigra projecting to the putamen and caudate of the brain. There are numerous contradicting results concerning the type of cell death in PD, but signs of activated apoptotic process were detected in the nigro-striatum of parkinsonian brains [1, 2]. Mitochondria have been considered to contribute to the development and progression of PD

either though the dysfunction of the complex I with subsequent reduced ATP synthesis and/or increased oxidative stress [3, 4]. However, recently direct involvement of mitochondria in neuronal apoptosis has been indicated [5, 6]. Various insults including oxidative stress, metabolic compromise and neurotoxins, induce opening of mitochondrial permeability transition (PT) pore with loss of membrane potential, $\Delta\Psi m$. The exact composition of PT remains to be characterized, but it is composed of voltage-dependent anion cannel, adenine nucleotide translocator, cyclophilin, creatine kinase, porin and hexokinase [7, 8]. Mitochondrial PT pore is regulated by bcl-2 family protein, either in suppressive (Bcl-2, Bcl-xL), or promoting way (Bax, Bad) [9, 10]. PT induces release of cytochrome c, apoptosis inducing factor and Ca^{2+} from mitochondria to cytoplasm, which promotes cleavage and activation of procaspase 9, followed by that of effecter caspases, procaspase-3 and procaspase-7. Finally nuclear DNA undergoes strand cleavage and condensation.

The type A and B of monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC. 1.4.3.4, MAO] are located in mitochondrial outer membrane of neuronal, glial and other cells. MAO catalyzes the oxidative deamination of monoamine neurotransmitters, dopamine, norepinephrine and serotonin, and regulates their levels in the brain, indicating the important role in several neurological and psychiatric disorders [11, 12]. Oxidation of dopamine by MAO produces a cytotoxic reactive oxygen species (ROS), hydrogen peroxide, and dopaquinone, which cause inhibition of mitochondrial respiration, lipid peroxidation and finally cell death in PD [13, 14]. In addition, MAO, especially type B, oxidizes a protoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into an active toxin, 1-methyl-4-phenylprdinium ion (MPP^+), resulting in cell death of dopamine neurons in humans [15]. Inhibitors of MAO-B prevented MPTP-induced neuronal decline in primate [16], and delayed the progress of PD in humans [17, 18]. However, recent results from various points of view suggest that MAO may be involved directly in apoptotic process. In apoptosis induced by nerve growth factor withdrawal in PC12 cells, MAO expression was increased and identified as a potential target of pro-apoptotic signaling of p38 mitogen-activated protein (MAP) kinase [19]. Figure 1 shows the hitherto proposed role of MAO in the neuronal cell death in PD. Inhibition of oxidative phosphorylation by ROS and cytotoxic MPP^+ and quinone, induces necrosis, whereas mitochondrial PT leads to apoptosis though activation of death cascade.

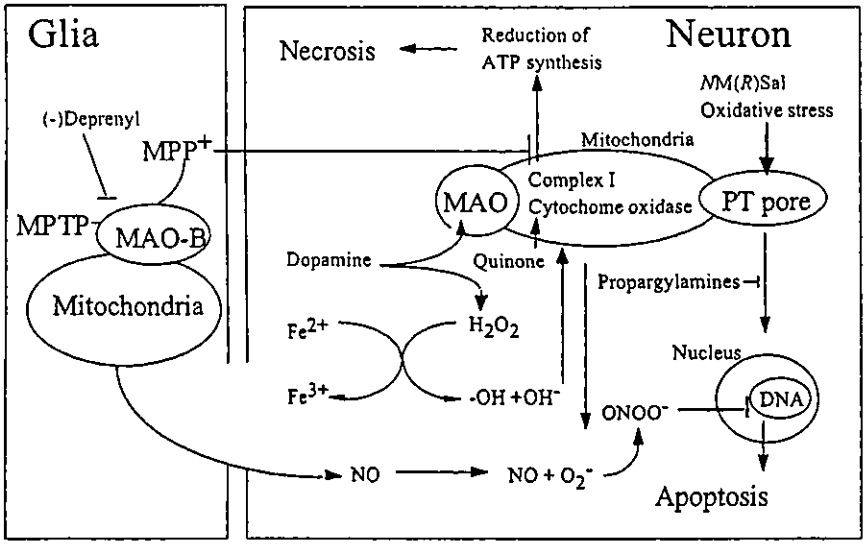
We found that (–)-deprenyl and related MAO-B inhibitors, including rasagiline [*N*-propaygyl-1(*R*)-aminoindan], *N*-(2-heptyl)-*N*-methylpropargylamine [(*R*)-2-HMP] and other propargylamines, enantio-selec-

tively protect dopamine neurons against apoptosis induced by neurotoxins, 6-hydroxydopamine (6-OHDA) [20], *N*-methyl(*R*)salsolinol [1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydropyridine, *N*M(*R*)Sal] [21–24], and nitric oxide and peroxynitrite [25]. These results suggest that compounds with affinity to the MAO-A binding site may be cytotoxic, whereas those to MAO-B may be related to neuroprotection.

Can MAO-A and MAO-B be involved in the decision of cell death either in promoting or inhibiting way though regulating mitochondrial death machinery? To prove our hypothesis, we examined the role of MAO in apoptosis induced by *N*M(*R*)Sal, a MAO-A inhibitor, and neuroprotection by propargylamine MAO-B inhibitors. This review summarizes our recent results in concern to new function of MAO regulating cell death in neurodegenerative disorders.

Fig. 1

Involvement of MAO in cell death in Parkinson's disease. In neurons MAO oxidizes dopamine to produce hydrogen peroxide and autoxidation to dopaquinone, which inhibit mitochondrial complex I directly or indirectly though oxidative modification and lipid peroxidation. In glial cells MAO-B oxidizes MPTP to MPP$^+$, which is taken up by dopamine transport system in neurons, where it inhibits complex I. Mitochondrial dysfunction increases superoxide, which reacts with nitric oxide to produce peroxynitrite. Peroxynitrite damages nuclear DNA directly. (–)Deprenyl prevents oxidation of MPTP to MPP$^+$, whereas propargylamines suppress opening of PT pore.



Endogenous isoquinolines as inhibitors of MAO-A

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, Sal) was for the first time detected in urine of parkinsonian patients treated with L-DOPA [26]. In human tissues three classes of monoamine-derived alkaloids have been reported; dopamine-derived 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines (catechol TIQs), and β-phenethylamine-derived TIQs and indolamine-derived β-carbolines [27, 28]. The non-enzymatic Pictet-Spengler reaction produces Sal derivatives from dopamine with aldehydes or keto-acids. However, the predominant occurrence of the (*R*)enantiomers of Sal was confirmed in human urine [29, 30] and the brain [31]. It suggests the enzymatic synthesis of Sal *in situ*, and the enzymes for the biosynthesis were isolated from human brains. (*R*)Salsolinol synthase catalyzes the enantio-specific synthesis of (*R*)salsolinol from dopamine and acetaldehyde [32], and (*R*)salsolinol *N*-methyltransferase synthesizes *N*M(*R*)Sal [33], which is further oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion [DMDHIQ$^+$] by non-enzymatic and enzymatic oxidation [34]. The step-wise reactions, *N*-methylation and oxidation, induce the specified distribution of *N*M(R)Sal and DMDHIQ$^+$ in the nigro-striatum of human brain [35].

Sal derivatives were relatively weak, but selective and competitive inhibitors of MAO-A [36, 37], and the (*R*)-enantiomers are more potent than the (*S*)-enantiomers [38]. The structure-activity relationship of Sal as MAO inhibitors was reported in details previously [39, 40]. The effects of naturally occurring catechol isoquinolines on activities of MAO-A and B were examined using mitochondria prepared from human brain synaptosomes, and the results are summarized in Table 1. The (*R*)-enantiomers of Sal and *N*MSal inhibited MAO-A more potent than the (*S*)enantiomers, and the inhibition was competitive to the substrate, whereas that of MAO-B was noncompetitive, as shown in Figure 2 [41]. The presence of hydroxyl groups at 6 and 7 position and substitution of a hydrogen group at the first position with a methyl or dihydroxybenzyl group is required for the inhibition, whereas the presence of a carboxy group at this position depletes the inhibitory activity. The oxidized DMDHIQ$^+$ is the most potent inhibitor of MAO-A [42].

The enantio-selective neurotoxicity of *N*-methyl(*R*)salsolinol

The induction of parkinsonism in humans by MPTP [43] suggests that endogenous and xenobiotic neurotoxins may be pathogenic factors of PD.

Table 1

The Ki value of salsolinol derivatives to type A and B MAO in human brain synaptosomes

Salsolinol derivatives	Ki values (μM) for monoamine oxidase	
	Type A	Type B
(*R*)Salsolinol	37.9	68.3
(*S*)Salsolinol	149.5	149.5
2(*N*)-Methyl(*R*)salsolinol	36.1	433.3
2(*N*)-Methyl(*S*)salsolinol	81.3	No inhibition
Norsalsolinol	No inhibition	No inhibition
2(*N*)-Methynorsalsolinol	61.4	289
1,2(*N*)-Dehydrosalsolinol	322.3	No inhibition
1-Carboxyl-(*R*)salsolinol	421.3	No inhibition
1,2(*N*)-DM-6,7-DHIQ$^+$ *	9.21	No inhibition
2(*N*)-M-6,7-DHIQ$^+$ **	44.4	No inhibition
2(*N*)-M-1-carboxyl(*R*)salsolinol***	No inhibition	No inhibition

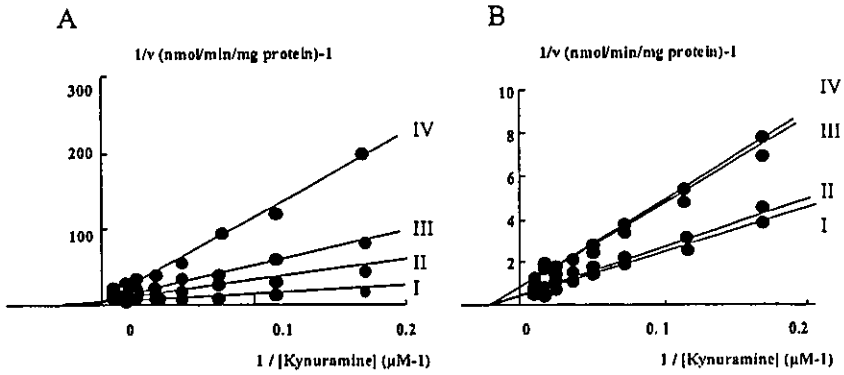
*, ** 1,2(*N*)-Dimethyl- and 2(*N*)-methyl 6,7-dihydroxyisoquinolinium ion
*** 2(*N*)-Methyl-1-carboxyl(*R*)salsolinol

The biosynthesis of Sal derivatives from dopamine and acetaldehyde suggests their involvement in pathological features of dopamine- or alcohol-related neurodegenerative disorders, such as PD and alcoholism. We examined the cytotoxicity of Sal derivatives to nigro-striatal dopamine neurons by the injection in the rat brain striatum [44]. *N*M(*R*)Sal induced parkinsonism in rats with reduction of dopamine levels and selective degeneration of dopamine neurons in the substantia nigra, proving this rat model to be adequate as a PD model. On the other hand, *N*M(*S*)Sal and other Sal derivatives did not cause any biochemical, behavioral and histo-pathological changes in rats. Involvement of *N*M(*R*)Sal in the pathogenesis of PD was suggested by significantly high level of *N*(*R*)Sal in the cerebrospinal fluid [45], and of (*R*)Sal *N*-methyltransferase activity in lymphocytes from parkinsonian patients [46].

*N*M(*R*)Sal induced apoptosis in dopamine neurons and the mechanism of cell death was studied using human dopaminergic neuroblastoma SH-SY5Y cells [22–24, 47, 48] Among catechol isoquinolines, *N*M(*R*)Sal

Fig. 2

The effects of Sal derivatives on MAO-A and B activity.
MAO-A and B were prepared from human brain synaptosomes. A: MAO-A activity. B: MAO-B activity. The reciprocal of the reaction velocity was plotted against that of the substrate, kynuramine, concentration. I: Control, II; +100 μM (*R*)Sal, III; *N*M(*R*)Sal, IV; DMDHIQ$^+$.



was the most potent to induce DNA damage, whereas the (*S*)enantiomer, Sal and DMDHIQ$^+$ were less apoptogenic, as summarized in Table 2. Figure 3 shows that *N*M(*R*)Sal induced cell death in SH-SY5Y cells, and most of dead cells were apoptotic, as shown by condensed and fragmented nuclei stained with Hoechst 33342. The intracellular mechanism underlying the apoptosis induced by *N*M(*R*)Sal was clarified as shown in Figure 4. The neurotoxin induced mitochondrial PT with collapse in membrane potential, $\Delta\Psi m$, followed by release of cytochrome C, activation of caspase 3, nuclear translocation of glyceraldehydes-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate: NAD$^+$ oxidoreductase (phosphorylating), EC1.2.1.12, GAPDH], and finally condensation and fragmentation of nucleosomal DNA. Only the (*R*)-enantiomer of *N*MSal induced $\Delta\Psi m$ decline in SH-SY5Y cells, and the (*S*)-enantiomer did not [22], as shown in Figure 5. The enantio-specificity of the (*R*)-enanitomer was further confirmed by induction of $\Delta\Psi m$ decline and swelling in isolated mitochondria [24].

These results suggest the presence of proteins in mitochondrial outer membrane, which distinguish the three-dimensional structure of the (*R*)-configuration of *N*M(*R*)Sal and induce mitochondrial PT.

Table 2

Cytotoxicity of salsolinol derivatives to SH-SY5Y cells

Salsolinol derivatives	Apoptotic cells (% of the total cells)	Necrotic cells
Control	4.86 ± 4.34	1.21 ± 1.68
(*R*)Salsolinol	9.55 ± 2.26	1.70 ± 1.41
(S)Salsolinol	10.71 ± 4.54	1.33 ± 0.68
N-Methyl(*R*)salsolinol	100*	0
N-Methyl(R)salsolinol*	26.73 ± 4.57#	0.85 ± 0.68
N-Methyl(*S*)salsolinol	10.9 ± 3.04	0.28 ± 0.44
DMDHIQ*	28.02 ± 9.09#	1.58 ± 1.8
(*R*)-1-Carboxy-Sal	3.73 ± 1.75	1.08 ± 1.10
(*S*)-1-Carboxy-Sal	5.72 ± 2.41	1.01 ± 0.78
Norsalsolinol	34.06 ± 5.05#	1.63 ± 1.68
N-Methylnorsalsolinol	8.87 ± 0.73	0.13 ± 0.32

The apoptotic and necrotic cells were classified by morphological observation after staining with Hoechst 33346.
The concentrations used were 500 μM, except *, 250 μM.
The number represents mean ± SD of three independent experiments.
Difference from control is statistically significant, $p < 0.01$.

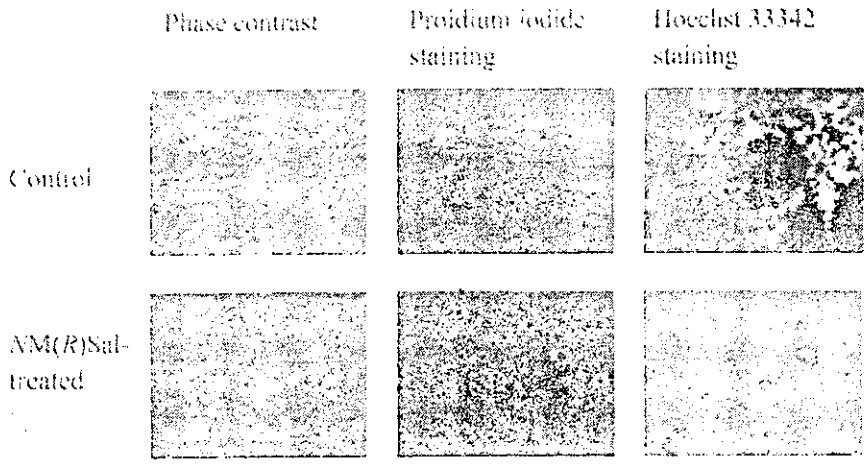
Neuroprotection by propargylamine MAO-B inhibitors

Recently, "neuroprotective" therapy has been proposed to attenuate the progressive decline of distinct neurons in neurodegenerative disorders [49, 50]. Candidates of neuroprotective agents are neurotrophic factors, anti-oxidant, radical scavengers, dopamine agonists, inhibitors of nitric oxide synthase and immuno-suppressors. A series of propargylamine derivatives related to (–)-deprenyl have been reported to prevent cell death of dopamine neurons in animal and cellular models of PD [51–53]. The intracellular mechanism underlying neuroprotection by propargylamines was studied using a cellular model of apoptosis induced by *N*M(*R*)Sal in SH-SY5Y cells. Figure 6 shows the chemical structure of propargylamines, whose anti-apoptotic capacity was confirmed.

Propargylamines protect the cells from apoptosis induced by the toxin, and the anti-apoptotic potency depends on the enantiomeric properties; the (*R*)-enantiomers were more potent than the (*S*)-enantio-

Fig. 3

NM(*R*)Sal induced apoptotic nuclear damage in SH-SY5Y cells.
The cells were treated with 500 μM of NM(*R*)Sal for 18 hours. Propidium iodide (PI) stained almost all the NM(*R*)Sal-treated cells. The nuclei were stained with a membrane-permeable dye, Hoechst 33342. Most of the nuclei in the toxin-treated cells were condensed and fragmented, as typical markers for apoptosis.

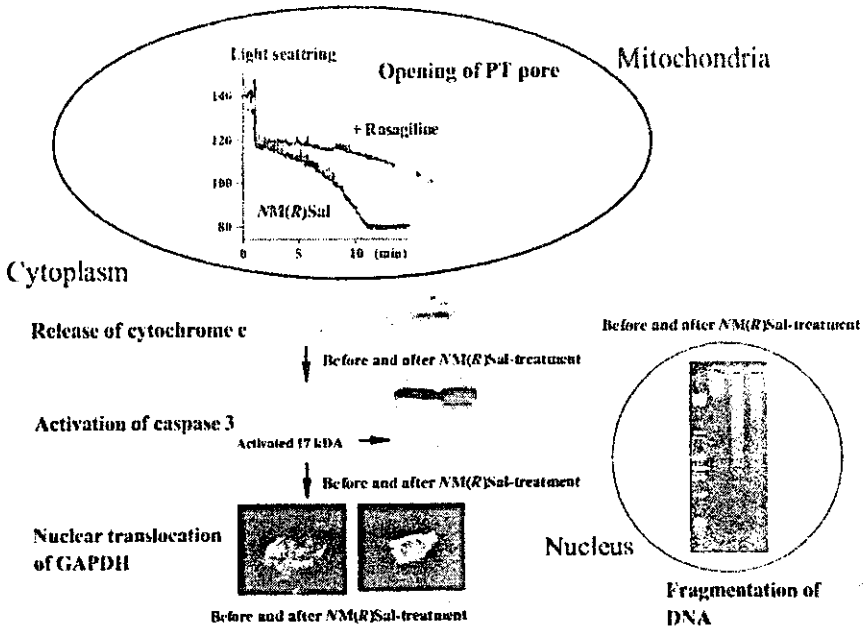


mers, as summarized in Table 3. Rasagiline and (*R*)-2-HMP prevent the ΔΨm decline in SH-SY5Y cells [21, 22] and in mitochondria prepared from rat liver (Figure 4) [24]. Rasagiline prevents the activation of following apoptotic signals: release of cytochrome c, activation of caspase 3, nuclear translocation of GAPDH and finally fragmentation of nuclear DNA, indicating that prevention of PT is primary for the anti-apoptotic function. The study on structure-activity relationship of propargylamines reveals that a propargyl residue is absolutely required for the stabilization of ΔΨm and the (*R*)-enantiomers are more potent to suppress the PT than the (*S*)-enantiomers [55].

These results suggest that a propargylamine residue should bind to protein closely associated with PT pore in the mitochondrial outer membrane. The detailed mechanism remains to be elucidated, but anti-apoptotic Bcl-2 protein seems to mediate the pro-survival function.

Fig. 4

Apoptotic cascade induced by *NM(R)*Sal.
***NM(R)*Sal induces mitochondrial PT in isolated mitochondria, as shown by swelling, then cytochrome C is released into cytoplasm in SH-SY5Y cells. Caspase 3 is activated, as shown by appearance of the active form with 17 kDa. Simultaneously GAPD is translocated into nuclei, as detected with anti-GAPDH antibody. Finally nuclear DNA is cleaved to fragments, as shown by the ladder formation.**



The involvement of MAO in the apoptotic process: some evidences supporting our hypothesis

Recent several papers suggest the involvement of MAO in apoptosis, but most of the results were indirect: MAO-A mRNA increased during apoptosis induced by NGF-withdrawal in PC12 cells [19], and MAO-B gene was induced in apoptosis by Galectin-7 (PIG1) in HeLa and DLD-1 cells [56]. We also found that rasagiline treatment increased MAO-A mRNA level in SH-SY5Y cells by two-folds. Previous papers interpreted the induction of MAO mRNA as increased of ROS produced by

Table 3

Anti-apoptotic activity of propargylamines

Compounds	Concentration required for the effect
(−)-Deprenyl (selegiline)	1 μM–100 nM
(+)-Deprenyl	10 μM
(−)-Desmethyldeprenyl	10–1 nM
(*R*)(+)-*N*-Propargyl-1-aminoindan (Rasagiline)	10–1 nM
(*S*)(−)-*N*-Propargyl-1-aminoindan (TV 1022)	1 μM–100 nM
Aminoindane	Not active
(*R*)-*N*-(2-Heptyl)-*N*-methylpropargylamine	1 μM–100 nM
(*S*)-*N*-(2-Heptyl)-*N*-methylpropargylamine	10 μM
(*R*)-3-(2-Heptylamine)-*N*-methylpropionic acid	Not active

SH-SY5Y cells were incubated with various concentrations of propargylamines for 20 min, then treated with 500 μM *N*M(*R*)Sal for 3 hours. Apoptotic cell death was quantitatively determined by "comet" assay, and the concentration required for the complete protection of the cells from apoptosis was obtained.

enzymatic oxidation of dopamine, as in the case with suppression of apoptosis by MAO-inhibitors [57, 58].

However, the results represented here strongly suggest that MAO may directly intervene apoptotic machinery, as shown by the enantioselective induction of PT by *N*M(*R*)Sal in isolated mitochondria and the cells. Recently we found that serotonin, a substrate of MAO-A, suppresses the $\Delta\Psi$m decline of isolated mitochondria induced by *N*M(*R*)Sal in a competitive way. On the other hand, clorgyline, an irreversible inhibitor of MAO-A, induced PT and apoptosis in SH-SY5Y cells. These results suggest that the binding site of neurotoxin and MAO-A substrates may be a common protein with binding site similar to that of MAO-A in mitochondria. MAO-A is detected predominantly in the substantia nigra, locus coeruleus, striatum and other regions containing catecholamine neurons, suggesting possible involvement of MAO-A in dopaminergic cell death in PD. On the other hand, the binding of anti-apoptotic propargylamines is not limited to MAO-B but also MAO-A, which may be relevant with the fact that MAO inhibitors has two different binding affinities; highly selective binding at nM order and low selective one at μM order.

Fig. 5

Changes of Ψm in the cells treated with *N*MSal.
SH-SY5Y cells were incubated in the absence or presence of 250 μM of *N*M(*R*)Sal or *N*M(*S*)Sal for 3 hours. Phase-contrast microscopy and the fluorescence microscopy after staining with JC-1. Red fluorescence represents *J*-aggregates insides intact mitochondria. The fluorescence decreased in the cells treated with *N*M(*R*)Sal without morphological change shown by phase-contrast microscopy. *J*-Aggregate fluorescence did not change in the cells treated with *N*M(*S*)Sal.

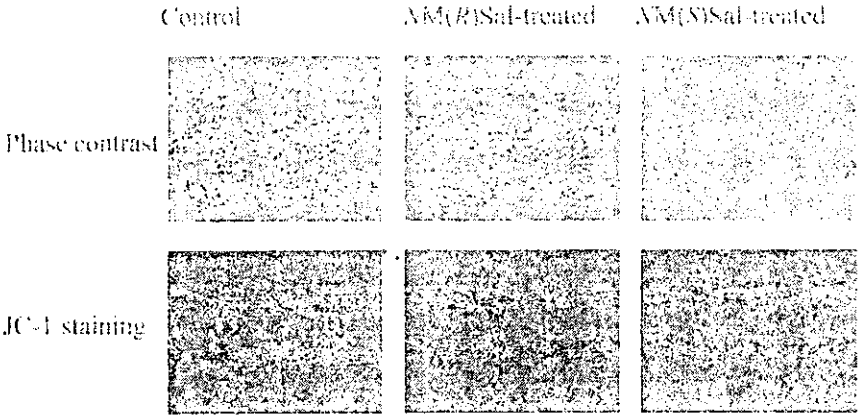
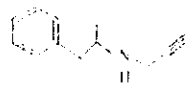


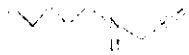
Fig. 6

Chemical structure of propargylamines with anti-apoptotic capacity.



(−)Deprenyl (selegiline)

N-Propargyl-1(*R*)-aminoindan
(Rasagiline)



N-(2-Heptyl)-*N*-methylpropargylamine
(2-HMP)

In this review, we propose our hypothesis about a novel function of MAO-A and MAO-B, as regulators of cell death and survival. We are now working further on this subject and we hope to be able to present more concrete evidences in near future.

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Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine in Rats

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ABSTRACT: **Methamphetamine, a psychostimulant drug, produces both acute psychomotor stimulation and long-lasting behavioral effects including addiction and psychosis. To identify anatomical substrates for the discriminative stimulus effects of methamphetamine in rats, we examined the drug discrimination-associated c-Fos expression in the brains of rats that were trained to discriminate methamphetamine from saline under a two-lever fixed ratio (FR-20) schedule of food reinforcement. c-Fos expression in the brains of rats trained to discriminate methamphetamine from saline was significantly increased in the nucleus accumbens (NAc) and the ventral tegmental area (VTA) as compared with the expression in the control rats that were maintained under the FR-20 schedule, but no alternation was observed in other areas including the cerebral cortex, caudate putamen, substantia nigra, hippocampus, amygdala, and habenulla. Methamphetamine treatment in the trained rats caused a significant increase in c-Fos expression in the VTA, and a decrease in the NAc core, as compared to saline treatment. However, c-Fos expression in the NAc and VTA of rats that received chronic intermittent methamphetamine administration without discrimination training, did not differ from the expression in saline-treatment animals. These results suggest that the VTA and the NAc play an important role in the discriminative stimulus effects of methamphetamine.**

KEYWORDS: **drug dependence; drug discrimination; c-Fos; methamphetamine; nucleus accumbens; ventral tegmental area**

INTRODUCTION

Methamphetamine (MAP), an addictive drug, produces various behavioral effects that are mainly mediated by the dopaminergic (DA) neuronal system.[1] It has been known that the discriminative stimulus effects of psychostimulants in experi-

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mental animals are related to their subjective effects in humans.[2] Therefore, the drug discrimination procedures have been proven to be a valuable means for elucidating the mechanism of action underlying the unique properties of addictive drugs.[1,3]

Quantification of the changes in the expression of the immediate early gene *c-fos* has been proven to be a very useful method by which the distribution of neurons that are activated by physiological or pharmacological stimuli may be mapped.[4] Several studies have shown that acute methamphetamine administration dose-dependently produces c-Fos-like immunoreactivity in wide areas of the brains including the nucleus accumbens and striatum,[5] and that chronic methamphetamine or amphetamine administration abolishes the inducibility of *c-fos* in the striatum.[6,7] In the present study, to identify potential anatomical substrates of the discriminative stimulus effects of methamphetamine in rats, we examined the c-Fos expression in the brain of rats trained to discriminate methamphetamine from saline.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (7 weeks old, Charles River Japan, Yokohama), weighing 230 ± 10 g at the beginning of experiment, were used in this study. They were under controlled laboratory conditions (12-h light/dark cycle with lights on at 9:00 h, 23 ± 0.5°C, 50 ± 0.5% humidity). Their body weights were gradually reduced to approximately 80% of the free-feeding weight by limiting daily access to food. Water was available *ad libitum*. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Methamphetamine Discrimination Procedure

Methamphetamine discrimination procedures were conducted as described in detail by Mori *et al.*[3] Rats were injected 10 min before the session with either saline or methamphetamine (0.5 mg/kg, s.c.). Discrimination training sessions were conducted 5 days per week under a double-alternation schedule (i.e., MMSSMMSS, etc., where M is methamphetamine; S, saline).

c-Fos Immunohistochemistry

A total of 44 of rats were prepared. Four groups were prepared in order to investigate the neural circuitry underlying the discriminative stimulus effects of methamphetamine: (1) naive rats that were subjected to neither food restriction nor drug discrimination training, (2) control rats that were maintained on the FR-20 schedule of food reinforcement without drug discrimination training, and (3) saline- and (4) methamphetamine-injected trained rats that had met the criteria for learning methamphetamine discrimination. Control rats were subjected to the FR-20 schedule of food reinforcement, while saline- and methamphetamine-injected rats were subjected to the test session of methamphetamine discrimination. Accordingly, the three groups of animals except naive rats obtained the same number (20 pellets) of food reinforcement by almost equal numbers of lever pressing. The saline- and methamphetamine-injected rats had the same drug history during the drug discrim-

ination training sessions, but received different drug treatment (methamphetamine vs. saline) on the test day. Rats were killed 2 h after the drug discrimination test for c-Fos immunohistochemistry. An additional four groups of rats were prepared in order to examine the effects of acute and chronic intermittent methamphetamine treatment on c-Fos expression—two groups of rats received chronic intermittent methamphetamine treatment at a dose of 0.5 mg/kg under a double-alternation schedule (i.e., MMSSMMSS etc., where M is methamphetamine; S, saline) without discrimination training. On the final day, half of these rats were challenged by methamphetamine 0.5 mg/kg or saline, respectively, and killed 2 h after the challenge. Another two groups of rats were injected with saline daily to examine the acute effects of methamphetamine. On the final day, half of the rats were injected with methamphetamine 0.5 mg/kg, while the others were treated with saline and killed 2 h after the treatment. Three separate groups of rats were treated with single saline or methamphetamine (0.5 mg/kg or 2 mg/kg, s.c.) to examine the dose-dependent effects of acute methamphetamine treatment on c-Fos expression, and killed 2 h after the treatment.

The c-Fos immunohistochemical procedure was the same as previously described methods.[8] Quantitative analysis of c-Fos immunohistochemistry was conducted by a computer-assisted image analysis system (C. Imaging Systems; Compix Inc., Mars, PA) as described previously.[9]

RESULTS

c-Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine

c-Fos expression were observed in all examined areas of the brain. Significant differences in c-Fos expression among the four groups of rats were observed in four brain areas including the cingulate cortex, the core and shell of NAc, and VTA (TABLE 1). There was a marked difference in c-Fos expression between control and trained groups in the core and shell of the NAc, and the VTA. Moreover, the number of c-Fos–positive cells was significantly smaller in the NAc core of methamphetamine-injected trained rats than in that of saline-injected trained rats, whereas it was increased in the VTA of methamphetamine-injected trained rats compared with saline-injected trained rats. No alteration in c-Fos expression was observed in other brain areas examined.

Effects of Acute and Chronic Intermittent Methamphetamine Treatment on c-Fos Expression

No significant alteration of c-Fos expression was observed in rats treated with chronic intermittent methamphetamine (0.5 mg/kg), whereas the number of c-Fos–positive cells in the NAc core of acute methamphetamine-treated rats was significantly higher than that of saline-treated rats.

Single administration with methamphetamine (0.5 and 2 mg/kg) produced a dose-dependent and significant increase in the number of c-Fos–positive cells in the NAc core, while methamphetamine 2 mg/kg, not 0.5 mg/kg, produced a significant increase in the NAc shell and the VTA.

TABLE 1. c-Fos expression in the brain subregions of rats trained for methamphetamine discrimination

Brain area	Naive ($n = 3$)	Control ($n = 4$)	Saline ($n = 4$)	Methamphetamine ($n = 4$)
Cerebral cortex				
Cingulate	72.3 ± 2.8*	102.4 ± 5.9	110.0 ± 3.4	115.7 ± 6.9
Motor	54.1 ± 3.2	66.7 ± 4.5	68.3 ± 3.4	68.0 ± 1.9
Somotosensory	38.8 ± 3.5	48.7 ± 1.8	51.4 ± 1.2	49.2 ± 2.7
Nucleus accumbens				
Core	36.8 ± 1.2	50.6 ± 4.3	21.9 ± 10.9***	90.3 ± 6.7*,#
Shell	30.6 ± 3.0	33.6 ± 5.8	86.0 ± 7.9***	71.2 ± 4.3**
Ventral tegmental area				
	16.7 ± 1.3	21.0 ± 4.7	57.4 ± 7.1**	90.4 ± 5.9***,##

Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were subjected to the drug discrimination test after either saline or methamphetamine (0.5 mg/kg) treatment. Control rats were maintained under the FR-20 schedule of food reinforcement without drug discrimination training. Naive rats were subjected to neither food restriction nor the methamphetamine discrimination training. c-Fos expression in each area is indicated as the number of c-Fos-positive cells per mm^2. Each value represents the mean ± SE. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus control. $^{\#}P < .05$, $^{\#\#}P < .01$ versus saline.

DISCUSSION

It is well known that DA plays a major role in the discriminative stimulus effects of methamphetamine.[1] In the discrimination test in rats, DA uptake inhibitors and D1 or D2 receptor agonists substituted for methamphetamine, whereas their antagonists completely blocked the discriminative stimulus effects.[1] In the present study, we demonstrated immunohistochemically that development of the ability to discriminate methamphetamine from saline in rats is associated with increases in c-Fos expression only in the VTA and NAc. This activation is unlikely due to lever-press behavior or food reinforcement because c-Fos expression in these brain areas did not increase in the control group maintained on the FR-20 schedule of food reinforcement (TABLE 1). Further, it is also unlikely that the changes are due to chronic intermittent methamphetamine treatment because such treatment without discrimination training did not increase c-Fos expression in the VTA and NAc.

Dopaminergic projections from the VTA to the NAc are involved in investigatory behavior evoked by novel stimuli and the reinforcement of adaptive investigatory approaches evoked by naturally occurring rewards and by addictive drugs.[10–12] It has also been suggested that the NAc core is more important than the NAc shell in response-reinforcement learning[13] and in behavioral response to motivationally significant stiumuli in general.[14,15] Accordingly, our findings suggest that the development of methamphetamine discrimination is associated with a selective activation of the VTA-NAc, probably the dopaminergic neuronal system. Compared to the shell region, c-Fos expression in the NAc core is more extensive, indicating that the